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Sculpting the Molecular Weight of Lignin via Laccase

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SCULPTING THE MOLECULAR WEIGHT OF LIGNIN VIA LACCASE

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ABSTRACT

Laccase was applied in combination with syringic, vanillic, and 4-hydroxybenzoic acid to lignin-impregnated cellulosic fibers and to fibers of a high-kappa pulp. Measurement of the molecular weight of the re-isolated lignin and material isolated from the pulp fiber surface via a surface refining technique revealed that laccase was able to couple these compounds to fibers resulting in molecular weight increases.

INTRODUCTION

Laccase is a robust oxidoreductase enzyme that has been studied extensively for potential industrial applications in pulp and paper. Since laccase substrates include phenolic compounds, aminophenols, polyphenols, polyamines, certain inorganic ions, and aryl diamine (1) compounds, it is a prime candidate for oxidizing lignin in pulp. Although laccase is capable of polymerizing lignin (2), most research in this area has focused on using laccase to degrade lignin in kraft pulps.

Laccase has been shown to actively oxidize both phenolic and nonphenolic lignin moieties in the presence of mediator compounds (3). Due to the size and redox potential of the enzyme, the proposed role for the mediator compound is to allow access to lignin in the fiber wall and to oxidize nonphenolic lignin units (3). Previous work in our group has shown that the laccase-mediator (LMS) system could be applied to high-kappa kraft pulps for delignification (4). Recently, we have become interested in exploiting the polymerizing ability of laccase to change the surface properties of high-kappa kraft pulps.

Laccase has only recently been employed for fiber modification. Both Felby (5) and Viikari (6) have employed laccases for fiber modification of mechanical pulps to improve paper strength properties. Yamaguchi (7) reacted various phenols with laccase to form dehydrogenative polymers (DHP). After DHP formation, these authors reacted the DHP with thermomechanical pulps in the presence of peroxidase to graft the DHP to the TMP fibers. These authors saw a 100% increase in tensile strength after sheet formation via hot pressing.

High-kappa kraft pulps possess a high amount of surface lignin (8) rendering them amenable to treatment with laccase enzymes. Recently we have shown that laccase could be used to polymerize phenolic compounds to high-kappa kraft pulps (9). Treatment of high-kappa kraft pulp with laccase and 4-hydroxybenzoic acid resulted in a 20% increase in kappa number (Figure 1) and an 80% increase in surface carboxylic acid groups according to Electron Spectroscopy for Chemical Analysis measurements (ESCA) (Figure 2) (9). Although these results support laccase–facilitated grafting of phenolic compounds to the fiber surface, there is no strong indication that the coupling is occurring on lignin.

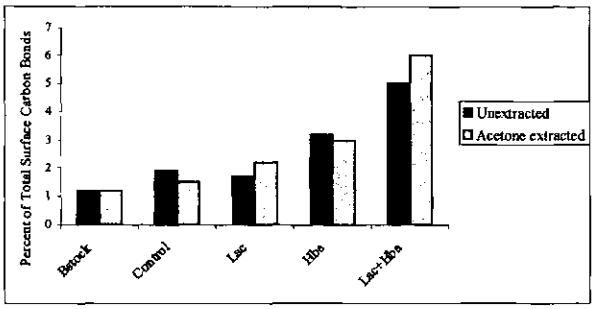


Figure 1: Surface Carboxylic Acid Group Measurement of Handsheets from High-Kappa Kraft Pulp Fibers Treated with Laccase and 4-Hydroxybenzoic Acid (9)

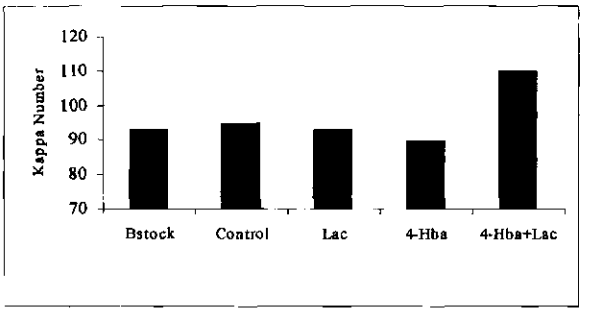
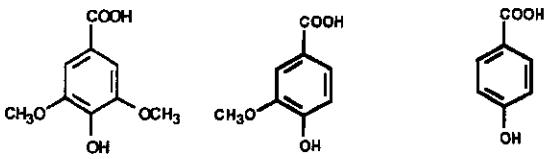


Figure 2: Results of Kappa Number Measurements of High-Kappa Kraft Pulp Treated with Laccase and 4-Hydroxybenzoic Acid (9)

Lignin-impregnated cellulosic fibers were prepared to elucidate the mechanism behind laccase-facilitated grafting of phenolic acids to high-kappa kraft fibers. Nuclear magnetic resonance analysis of the lignin provided strong evidence for fiber lignin being the site of coupling for the phenolic acids (9). In this work, syringic, vanillic, and 4-hydroxybenzoic acid (Figure 3) were reacted with laccase in the presence of lignin-impregnated fibers and high-kappa kraft pulp fibers for subsequent molecular weight analysis.



Syringic Acid Vanillic Acid 4-Hydroxybenzoic Acid

Figure 3: Phenolic Acids Employed in this Study

MATERIALS AND METHODS

Materials

All materials used in this study were purchased from Aldrich Chemical Co., Milwaukee, WI, and used as received except for *p*-dioxane. *p*-Dioxane was distilled over $NaBH_4$. Laccase from *Trametes villosa* was donated by Novo Nordisk Biochem, Franklinton, NC. Linerboard softwood kraft pulp was obtained from a commercial facility located in the southeastern U.S.A. The commercial pulp was exhaustively washed until the filtrate was pH neutral and colorless. Pulp was air-dried and Soxhlet-extracted for 24 hrs with acetone with subsequent washing with water prior to all treatments and lignin isolation procedures.

Methods

Laccase. The activity of laccase was measured by monitoring the rate of oxidation of syringaldazine. The change in A_{530nm} of 0.001 per minute per mL of enzyme solution in a 100-mM potassium phosphate buffer (2.2 mL) at pH 4.5 and 0.216 mM syringaldazine in methanol (0.300 mL) was set to one unit (U) of activity. This test was done at 23°C.

Lignin Isolation Procedure. Residual lignin was isolated from acetone-extracted, never-dried, softwood kraft pulp with a starting kappa of 90.5 following established literature methods (10). In brief, a three-necked round-bottom flask was charged with 50.0 g of pulp (o.d. basis). The consistency was adjusted to 4.00% by adding a 0.100 N HCl 9:1 *p*-dioxane:water solution. Next the slurry was refluxed for two hours under an argon atmosphere. The pulp was then filtered and the filtrate was filtered through celite, neutralized, and concentrated under reduced pressure to approximately 10% of the original volume. Water (approx. 200 mL) was added and the mixture was concentrated again under reduced pressure to remove the last vestiges of *p*-dioxane. The pH of the lignin solution was then adjusted to pH 2.5 with 1.00 N HCl. The precipitated lignin was collected, washed three times with dilute HCl at pH 2.5, and then freeze-dried (10).

Lignin Impregnated Cellulosic Fibers. Filter paper (5.00g of no. 4 Whatman) was fiberized in a Waring blender for 1.00 min. These fibers were combined with 200 mg of lignin and suspended at 15.0% csc in dioxane in a round-bottom flask. The dioxane was removed gradually under reduced pressure at 30°C. This yielded cellulose fibers impregnated with lignin.

Reaction of Lignin Impregnated Cellulosic Fibers with Laccase and Phenolic Acids. The reaction sequence is shown in **Table 1.**

Table 1: Treatment Regime Applied to High-Kappa Kraft Pulp Fibers and Lignin Impregnated Cellulosic Fibers

Treatment	Symbol
Brownstock	Bstock
Control	Control
Laccase+Pulp	Lac
Vanillic Acid + Pulp	Van
Vanillic Acid + Laccase + Pulp	Van + Lac
Syringic Acid	Syr
Syringic Acid + Laccase + Pulp	Syr + Lac
4-Hydroxybenzoic Acid + Pulp	4-Hba
4-Hydroxybenzoic Acid + Laccase + Pulp	4-Hba + Lac

Each phenolic acid (88.0 mg) was added to a Kapak bag containing the pulp slurry at 5.00% csc. The samples were pH adjusted to 4.50 and then brought to 45°C in a water bath. Laccase (2.00 mL) was added to the pulp mixture and the reaction was allowed to continue for 2.00 hours. Upon completion, each sample was washed with water until the filtrate was clear. The lignin was re-isolated from these fibers by soaking in 1:1 mixture of distilled *p*-dioxane and water for 12.0 hours and then filtering to remove the fibers. The filtrate was concentrated under reduced pressure and then combined with water for a final volume of 200 mL. The pH of the mixture was adjusted to 2.5 for acid precipitation of the lignin. These samples were then centrifuged for 25.0 min and decanted. Lignin samples were then Soxhlet-extracted for 2.00 hours with diethyl ether to remove physically bound monomers/polymers followed by air-drying. High-kappa (90.5) pulps were reacted with the same procedure as lignin-impregnated fibers except 25.0 g o.d. fiber was treated with a 6.00% charge of 4-Hba and laccase.

Acetylation of Lignin and Surface Material. Lignin samples were acetylated to solubilize in tetrahydrofuran (THF) or GPC analysis. A round bottom flask containing 20.0 mg of lignin was charged with 1.80 mL each of pyridine and acetic anhydride (1:1). The reaction mixture was allowed to stir for 24.0 hours at

room temperature. Ethanol (20.0 mL) was added and the solvents were removed under reduced pressure. This procedure was repeated 10 times for complete removal of pyridine and acetic anhydride. The acetylated lignin was dissolved in 10.0 mL chloroform and washed twice with de-ionized water. The sample was then dried over sodium sulfate for 24 hours. The lignin was then precipitated with anhydrous diethyl ether followed by centrifugation and removal of ether under reduced pressure. Surface material isolated from pulp was acetylated in the same procedure as lignin except methyl sulfoxide was added to the acetylation mixture. The mixture was heated to 70°C for 30.0 min prior to stirring for 48 hours.

Isolation of Surface Material from Pulp Fibers. Surface material from laccase-treated pulp fibers was isolated via a technique outlined by Heijnesson et al. (11). In brief, fibers were treated in a disintegrator at 1.5% csc (this csc was employed due to the size of the pulp sample in order to fill the disintegrator) for 110,000 revolutions resulting in an isolation of 0.45% of starting pulp material.

GPC Analysis. GPC analysis was performed according to the methods outlined in (12). Acetylated lignin samples were allowed to stir overnight in THF to fully solubilize followed by filtering through a 0.2-um Teflon syringe filter prior to injection. Samples (100 ul) were subjected to GPC analysis on a 2-column sequence of Waters™ Styragel columns (HR1/2) standardized with polystyrene standards and eluted at 1.00 mL per min.

RESULTS AND DISCUSSION

Laccase has been shown to possess the ability to polymerize lignin preparations (2). Furthermore, laccase has also been shown to graft foreign compounds to lignin in solution (13,14). Lund and Ragauskas (14) reacted lignin and laccase in solution with various phenolic monomers and found evidence for their incorporation into lignin in solution. These studies are beneficial to understanding laccase-facilitated graft polymerization with lignin. However, it is not known if the reactions of laccase and lignin in solution were representative of those with laccases and pulp fibers in suspension.

In the work described here laccase was reacted with water-soluble phenolic acids on a lignin-impregnated solid fiber to mimic the reaction occurring with real pulp fibers. As shown in Figure 4, laccase treatment with 4-hydroxybenzoic acid resulted in a slight increase in molecular weight. The results obtained from treatment of lignin-impregnated fibers with laccase+4-Hba indicated laccase-facilitated coupling of phenolic acids to lignin in the solid state. These results correlate with previous results when laccase and 4-Hba was the best combination for increasing kappa number of pulp fibers (Figure 1).

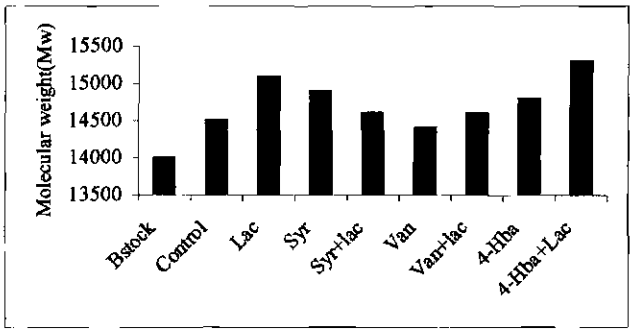


Figure 4: Molecular Weight of Lignin Re-isolated from Lignin Impregnated Fibers

The differences in reaction of the phenolic acids may be attributed to the presence of methoxyl groups in both syringic and vanillic acid, which may block potential sites for polymerization. Yamaguchi (15) reacted milled wood lignin (MWL) with a phenolic DHP of vanillic acid in the presence of peroxidase and found an increase in molecular weight of the MWL. It should be noted that these authors used a DHP to graft to the MWL. The resulting changes in molecular weight from binding of a DHP to MWL lignin would be easier to discern than the grafting of low molecular weight phenolic acids to the lignin macromolecule as shown in the work here. Since the increases in molecular weight were difficult to distinguish during treatment of lignin impregnated fibers, the laccase treated high-kappa fibers themselves were analyzed for molecular weight changes on actual fiber surfaces by utilizing a "surface refining" technique (11).

Heijnesson (11) et al. established the "surface-refining" method as a means for isolating surface material from kraft fibers. The surface material has been shown to contain a high proportion of lignin (11). Samples of high-kappa pulp (90.5 kappa) were treated with laccase and laccase + 4-Hba acid with subsequent surface refining to isolate surface material. The acetylated surface material was analyzed via gel permeation chromatography.

The data summarized on Figure 5 shows that surface refining isolated low molecular weight material (M_w 1000-3000 range). It is evident from these results that laccase treatment in the presence of 4-Hba acid resulted in a 100% increase in molecular weight of fiber surface material.

Laccase treatment in the absence of 4-Hba resulted in very little molecular weight change compared to the

samples treated with laccase and 4-Hba. Felby (5) described laccase polymerization of colloidal lignin and extractives with the fiber surface. The difference between the results here and those of Felby (5) may be attributed to the fact that the pulps treated here were kraft pulps extracted with acetone prior to treatment compared to the unextracted mechanical pulp fibers used by Felby. In summary, the results presented here strongly suggest laccase-facilitated coupling of phenolic acids to fiber surfaces. Future work will focus on further analysis of material obtained from surface via the surface refining technique employed here.

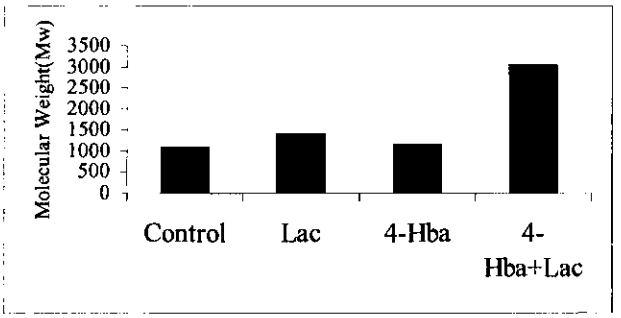


Figure 5: Molecular Weight Results of Material Isolated from Fiber Surfaces via Surface Refining Technique

CONCLUSIONS

The results presented indicate laccase-facilitated coupling of 4-hydroxybenzoic acid to pulp and lignin-impregnated fibers. The data represent evidence supporting laccase's ability to graft in a heterogenous reaction with soluble phenolic acids and solid fibers.

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